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Phase I clinical trial with a hexavalent PorA containing meningococcal outer membrane vesicle vaccine

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A meningococcal outer membrane vesicle (OMV) vaccine was prepared from two production strains designed to express three serosubtype-specific class 1 outer membrane proteins or PorA. The resulting hexavalent PorA OMV vaccine contained the serosubtypes P1.7,16; P1.5,2; P1.19,15; P1.7^h,4; P1.5^c,10; P1.12,13 and were used to immunize adult volunteers. A single immunization with two dosages, 7.5 and 15 μg of the individual PorAs, was studied. The vaccine was considered safe for further use. Approximately half of the volunteers demonstrated a fourfold increase in bactericidal antibody activity against six test strains expressing the specific PorAs when given the higher dosage. This bactericidal activity was found to be directed against PorA. Copyright © 1996 Elsevier Science Ltd.

Keywords: Meningococcal vaccine; outer membrane vesicle; PorA

We chose to develop a serogroup B meningococcal vaccine on the basis of the class 1 outer membrane protein (OMP) or PorA. The reasoning behind this choice relied upon findings such as the bactericidal activity and animal protection capacity of murine monoclonal antibodies[1–4]. In addition, mutant strains devoid of PorA poorly induced bactericidal antibodies in mice and transfer and expression of the P1.5,2 *porA* gene to a P1.7,16 strain induced a full bactericidal response against P1.5,2 strains. PorA negative strains can hardly be killed by polyclonal mouse sera induced with outer membranes[5]. Initially we developed a purified PorA, PorB, Rmp containing vaccine for human use[1]. The bactericidal antibodies induced in two of six adult volunteers were found to be directed against PorA (this paper). The other four did not develop an increase in bactericidal titers after two immunizations. Our results in mice suggest that outer membrane vesicles (OMV) are superior to purified OMP vaccines[2]. This can easily be explained by assuming that OMV preserve and expose the epitopes associated with surface loops in a native way[6]. The experience after large-scale field trials with OMP and OMV vaccines (from a single epidemic strain containing PorA, PorB, Rmp, and others) suggests that protection can be induced[7–9]. OMP vaccines did not appear very protective in infants[7–10]. It is crucial to appreciate if this relates to the formulation of the OMP vaccine and if well-characterized OMV vaccines not containing nonvesicular OMP will be able to induce a bactericidal immune response in infants. In addition, vaccines derived from single strains induce type-specific bactericidal antibodies for the greater part[11]. Because of the findings described above we developed multivalent PorA containing OMV vaccines for clinical testing[12,13].

Two vaccine strains were manipulated by rDNA technology to express three PorA proteins each, the homologous and two additional heterologous proteins. Each PorA protein expresses two surface exposed variable regions, both of which are targets for bactericidal antibodies[6]. The PorA protein contains therefore two serosubtype specific epitopes[14] which are named such as P1.7,16 and others, P1.7 being present within variable region 1 (surface loop 1 out of the eight predicted loops) and P1.16 within variable region 2 (surface loop 4). We constructed two vaccine strains with the following composition: P1.7,16; P1.5,2; P1.19,15 and P1.7^h,4; P1.5^c,10; P1.12,13. These serosubtypes represent the majority of case isolates in many countries[15]. An OMV vaccine was prepared from these strains leading to a hexavalent PorA vaccine containing 12 serosubtype epitopes. The vaccine was tested in adult volunteers for safety after one dose. The immunogenicity was measured by ELISA using OMV, western blotting and by bactericidal assay.

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MATERIALS AND METHODS

Purified OMP vaccine

After fermentor growth, strain H44/76 (B:15:P1.7,16) was extracted with Zwittergent-3,14 and the mixture of PorA, PorB, and Rmp was purified by ionic exchange and gel permeation chromatography. The vaccine was formulated with aluminumphosphate and meningococcal C polysaccharide and injected in a 0.5 ml volume[1]. Six adult volunteers were immunized twice with a 1-month interval using 25 μg total protein and 25 μg polysaccharide. The vaccine was studied in-house after approval by an ethics committee, and was conducted in 1987. The vaccines contained 1.5 mg $AlPO_4$ and 0.05 mg thiomersal and H_2O per dose in a volume of 0.5 ml.

Hexavalent PorA-OMV vaccine

The hexavalent meningococcal group B OMV vaccine was prepared as described[13]. The final vaccine contained an equal aliquot of the OMVs of the two recombinant engineered trivalent meningococcal strains PL16215 (−:P1.7,16; P1.5,2; P1.19,15) and PL10124 (−,$P1.5^c$,10; P1.12,13; $P1.7^h$,4) adsorbed to $AlPO_4$, containing sucrose as a stabilizer. The nonvaccine trivalent strain PL9146[12] (−:P1.9; P1.14; P1.6; variable region 1 not yet defined) served as a control. Three vaccines were tested: lot E 9282 (50 μg total protein); lot E 9281 (100 μg total protein) and lot E 9283 (placebo). The vaccines contained 1.5 mg $AlPO_4$; 10 mg sucrose; 0.05 mg thiomersal and per dose in a volume of 0.5 ml H_2O. PorA mounted to 90% of the vaccine protein, which implies 7.5 μg or 15 μg of each of the individual PorAs in a vaccine dose, respectively.

Phase I study protocol with the OMV vaccine

A phase I safety study was approved by the ethics committee of the Utrecht University Hospital (The Netherlands). Three groups of ten healthy adults each volunteered for the study. Group one was given a placebo being similar to the vaccines without OMV (lot E 9283), group two the vaccine containing 50 μg (lot E 9282), and group three the vaccine containing 100 μg (lot E 9281) total protein. The vaccine was given by injection in the deltoid muscle. The study was performed by the Department of Intensive Care I and Clinical Toxicology of the Utrecht University Hospital and the National Poison Control Center of the National Institute of Public Health and Environmental Protection (RIVM). The volunteers were selected by a check-in medical examination, consisting of a medical history and a physical examination, electrocardiography, and routine blood and urine investigation and the volunteers had to meet the admission criteria, as specified in the study protocol. Each volunteer was observed for 4 h following the injection. Blood pressure and heart rate were measured at 15 min, 30 min, 60 min, 2 h, and 4 h following the injection. Body temperature was measured before, and 2 and 4 h after injection. At regular intervals the volunteers were asked for any reactions and questionnaires were completed after 2 and 4 h following injection. After 2 days a check was performed including an assessment of adverse events by medical history, well being, measurement of body temperature, blood pressure and heart rate, physical examination, and blood and urine samples were taken for routine examination. After 14 days a check-out was done by the same procedure as by the intake. Investigation of adverse events or any other complaint was done, and blood and urine samples were taken for routine examination. Just before immunization and 14 days after vaccination blood was taken for immunological studies. The blood was allowed to clot for 30 min at room temperature and the serum was stored until use in small aliquots at −70°C.

Serology, ELISA

Antibody titers to OMV from the trivalent strains PL16215, PL10124, and PL9146[12] were determined by enzyme linked immunosorbent assay as described below. PL9146 was made according to the same procedures[12] and used as a control since it contains serosubtypes not included in the hexavalent vaccine.

High activated immunoassay microtiter plates (Tecnolab International, The Netherlands) were coated with 5 μg ml^{-1} of total protein of OMV in 0.01 M phosphate-buffered saline (PBS) pH 7.4. After overnight incubation at room temperature, plates were washed with tap water–Tween-80 (0.03%). All plates were then incubated for 90 min at 37°C with threefold serial dilutions of serum samples in PBS containing 0.1% (v/v) Tween-80. Plates were washed again and incubated for 90 min at 37°C with peroxidase conjugated rabbit anti-human IgG antibodies (Dakopatts, code P214) PBS–Tween-80 0.1% (w/v), and Skimmed milk powder 0.5% (w/v). Plates were washed again and incubated at room temperature with 100 μl of the peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (0.1 mg ml^{-1}; Sigma) with 0.01% H_2O_2 in 0.11 M sodium acetate buffer pH 5.5. After 10 min incubation the reaction was stopped by adding 100 μl of 2 M H_2SO_4. The A_{450} was registered on a Biokinetics microplate reader (Bio-Tek, EL312e). The data from each plate were analyzed using the KinetiCalc V2.03 calculation programme (Bio-Tek). IgG antibody titers in pre- and postvaccination serum samples were expressed as reciprocal dilution giving an absorbance of 0.8 at 450 nm.

Serology, serum bactericidal assay

Starting with H44/76 (B:15:P1.7,16) five isogenic strains, termed TR52, TR15, TR10, TR1213, and TR4 were derived by exchange of the *porA* gene with the same set of alleles as present in the trivalent vaccine strains. The gene donor strains used were 2996 (B:2b:P1.5,2), MC51 (C:nt:P1.19,15), 870227 (B:4:$P1.5^c$,10), 870446 (B:14:P1.12,13) and 892257 (B:4:$P1.7^h$,4), respectively. The *porA* genes of these strains were amplified by PCR, digested with restriction enzymes HindIII-KpnI and used for replacement of the corresponding part of plasmid pCO3, as described previously[12]. For strains 2996 and MC51, plasmids carrying a directly cloned chromosomal *porA* gene were used. Plasmid DNA was added to strain H44/76 and transformants resulting from *porA* gene replacement were identified by colony blot[5]. By verifying the absence of P1.7 and P1.16 epitopes, replacement of both variable regions was confirmed. All strains displayed a high level of class 1 protein expression, as shown by analysis of outer membrane complexes by SDS–PAGE. The strains were

Table 1 Bactericidal antibodies in adult volunteers immunized with purified meningococcal outer membrane proteins (1987)

Volunteer	Bactericidal test		
	H44/76	HI-5 (cl 1⁻)	HIII-5 (cl 3⁻)
No. 1			
Pre	20	<20	<20
Post 1	N.D.	N.D.	N.D.
Post 2	80	20	80
No. 2			
Pre	<20	<20	<20
Post 1	160	20	160
Post 2	80	20	160
No. 3 (4, 5, 6)			
Pre	20	<20	80
Post 1	20	<20	80
Post 2	20	<20	80

N.D.=not determined. The vaccine is described in Ref. 1

Table 2 Adverse events in adult volunteers after immunization with a hexavalent PorA containing meningococcal OMV vaccine (mild, unless specified)

	Placebo	50 μg vaccine	100 μg vaccine
Local[a]			
Redness	3	2	1[b]
Swelling	0	0	1
Warmth	1	0	1
Itching	0	1	1
Pain (local)	6	10[c]	10[d]
Systemic			
Headache	0	1	0
Gastro-enteritis	0	1[e]	0
Joint	0	0	0
Skin	0	0	0
Impairment	0	1[e]	0
Daily activities			
Sick	0	1[e]	1
$n = 10$		10	10

[a]Number of reporting participants: moderate, interfering with daily activities; severe, daily activities impossible. [b]Moderate; [c]one moderate, five severe; [d]four moderate, two severe; [e]same volunteer with gastro-enteritis

grown onto several GC agar plates containing 1% IsoVitalex. After overnight incubation at 37°C in 5% CO_2 the colonies were harvested with a sterile polyester swab and suspended in MHB with 15% glycerol. Working seed lots of 1.5 ml were stored at −70°C. A sample of this working seed lot was tested for expression of epitopes using monoclonal antibodies (see section on Monoclonal Antibodies Used). To eliminate phase variation a fresh aliquot was plated on GC agar plates with IsoVitaleX for each day's experiment. A scrape was taken from the frozen seed lot and streaked onto a GC agar plate with IsoVitaleX. After overnight incubation at 37°C in 5% CO_2 colonies were harvested in 2 ml of sterile MHB. Approximately 100–200 μl of the stock bacterial cell suspension was added to 20 ml of sterile MHB pre-equilibrated at room temperature to yield an A_{600} between 0.07–0.08. The culture flask was then incubated for *ca* 2 h at 37°C with 160 revs min^{-1} shaking until A_{600} was between 0.22–0.24. This yielded *ca* 1.10^9 c.f.u. ml^{-1}. The bacterial cells were diluted in sterile Gey's Balanced Salt Solution containing 0.5% (w/v) BSA (assay buffer) until a concentration of 1.10^5 c.f.u. ml^{-1} was reached (bacterial working concentration).

All human sera to be tested were heat inactivated for 30 min at 56°C. Plasma from an agammaglobulinemic patient with no bactericidal activity against the strains to be tested and having normal complement activity was used as a complement source in this assay. A sterile polystyrene U bottom 96 well microtiter plate was used for the microbactericidal assay. The total volume of each well of the plate was 50 μl with 25 μl of twofold serial diluted serum in assay buffer (final starting dilution 1:4), 12.5 μl of bacteria, 12.5 μl of complement [final concentration 10% (v/v) in assay buffer]. Controls included samples with buffer, bacteria and complement and samples with buffer, bacteria and heat inactivated complement. A known positive serum sample was included in each assay; the acceptable limit of variability was one well dilution in a twofold dilution series. After addition of the components to each well of the plate a 10 μl aliquot was taken from the control samples with buffer, bacteria and complement and pipetted onto a dry, square formed GC agar plate containing 1% IsoVitaleX and allowed to run in lanes down the plate. The microtiter plate was then incubated for 60 min at 37°C. The GC agarplate with 1% IsoVitaleX was incubated 18 h at 37°C in 5% CO_2. After 1 h incubation a 7 μl aliquot was taken from each well of the microtiter plate of a lane by using a multichannel pipet and spotted onto a dry, square formed GC agar plate containing 1% IsoVitaleX. After 18 h incubation at 37°C in 5% CO_2, the colonies from time zero and 60-min-incubation plates were counted. The average number of c.f.u. at time zero was set at 100%. The serum bactericidal titer was reported as the reciprocal of the serum dilution yielding ≥50% killing.

SDS–PAGE and western blotting

Outer membrane vesicle preparations of the trivalent strains PL16215 and PL10124 were separated on a 12% SDS–PAGE gel at constant amperage of 30 mA per gel for *ca* 2 h. Sample buffer was added to the OMV preparation and the mixture was heated for 5 min at 100°C. A total protein concentration of 80 μg protein was loaded on the top of the gel. After running the gels, the separated proteins were transferred to nitrocellulose sheets in a semi dry Electroblotter (Ancos) at a constant current of 0.8 mA cm^2 for 1 h. The nitrocellulose sheet was then cut into small strips of 3 mm. The nitrocellulose strips were incubated with a 1:400 dilution of pre- and postvaccination serum samples in PBS +0.5% Tween-80 (PBST). For control, strips were incubated with monoclonals recognizing the surface exposed loops P1.16, P1.2, P1.15, P1.10, P1.4, P1.13, class 4 protein, class 5 protein (5.5), and 5C protein. Subsequently, excess binding was blocked by incubation of the nitrocellulose strips in PBST and 0.5% (w/v) skimmed milk powder for 10 min at room temperature. After washing, the strips were incubated for 1 h at room temperature with either peroxidase-labeled goat anti mouse IgG (Southern Biotechnology Associates) or with peroxidase-labeled rabbit anti human IgG (Dakopatts,

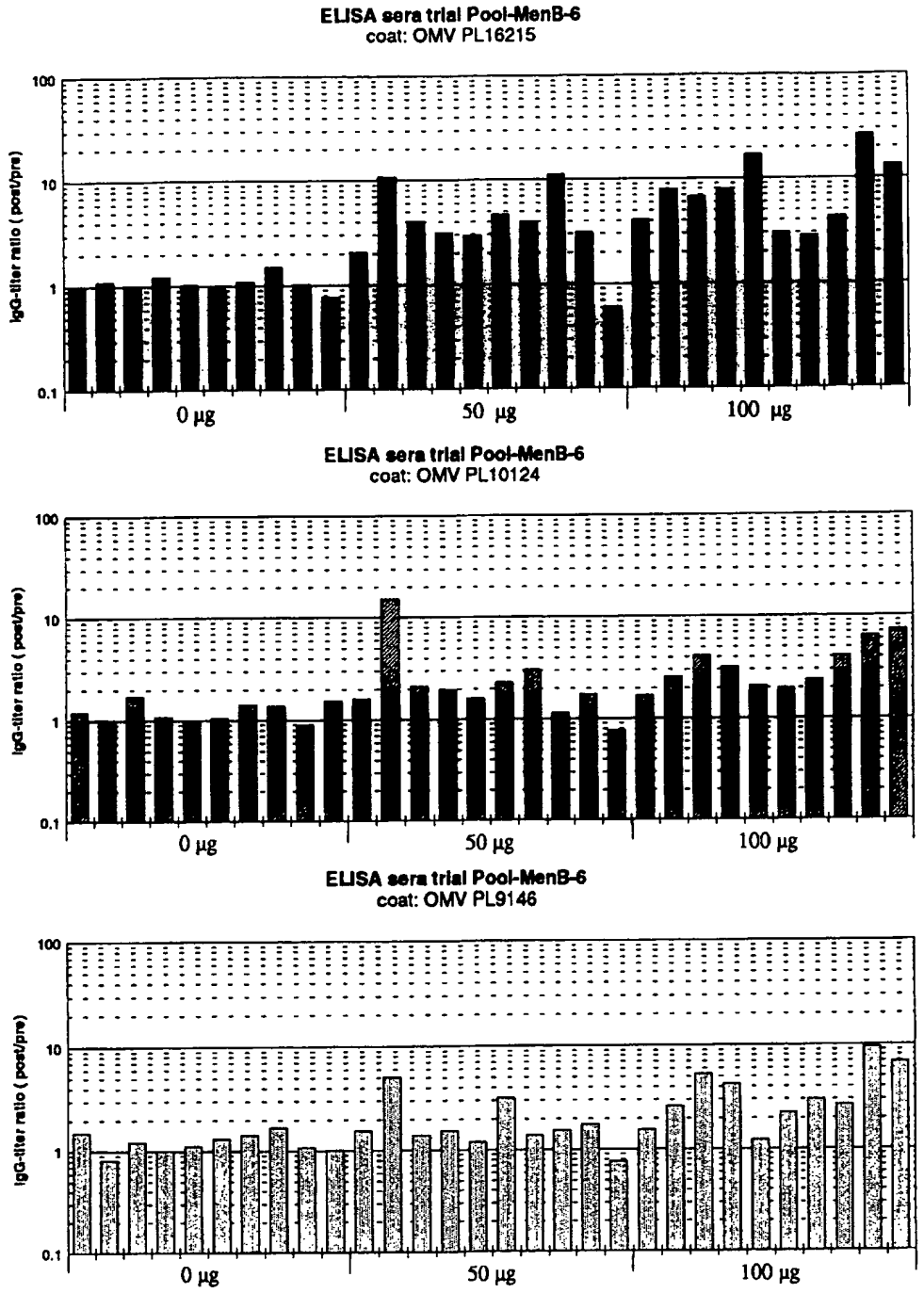

Figure 1 Antibody activity after one dosage of 50 or 100 μg hexavalent PorA meningococcal OMV vaccine in adult volunteers with respect to IgG using trivalent PL16215, PL9146, PL10124 OMV in ELISA

code P214) diluted in PBST with 0.5% (w/v) skimmed milk. The strips were washed again and subjected to coloring with substrate.

Monoclonal antibodies used

The following monoclonal antibodies were used: MN5C11G (anti-P1.16); MN16C13F4 (anti-P1.2); MN3C5C (anti-P1.15); MN2D6D (anti-class 4 OMP); S3571 (anti-5C protein, kindly given by Mark Achtman, Berlin); 15-1-P5.5 (anti-class 5.5 OMP, kindly given by Wendell Zollinger, Washington, DC); MN20F4.17 (anti-P1.10); MN24H10.75 (anti-P1.13); MN20B9.34 (anti-P1.4).

RESULTS

Six adult volunteers were immunized twice with a 1-month interval using a purified PorA (class 1 OMP), PorB (class 3 OMP), Rmp (class 4 OMP) meningococcal OMP vaccine[1]. No systemic or local side-reactions were

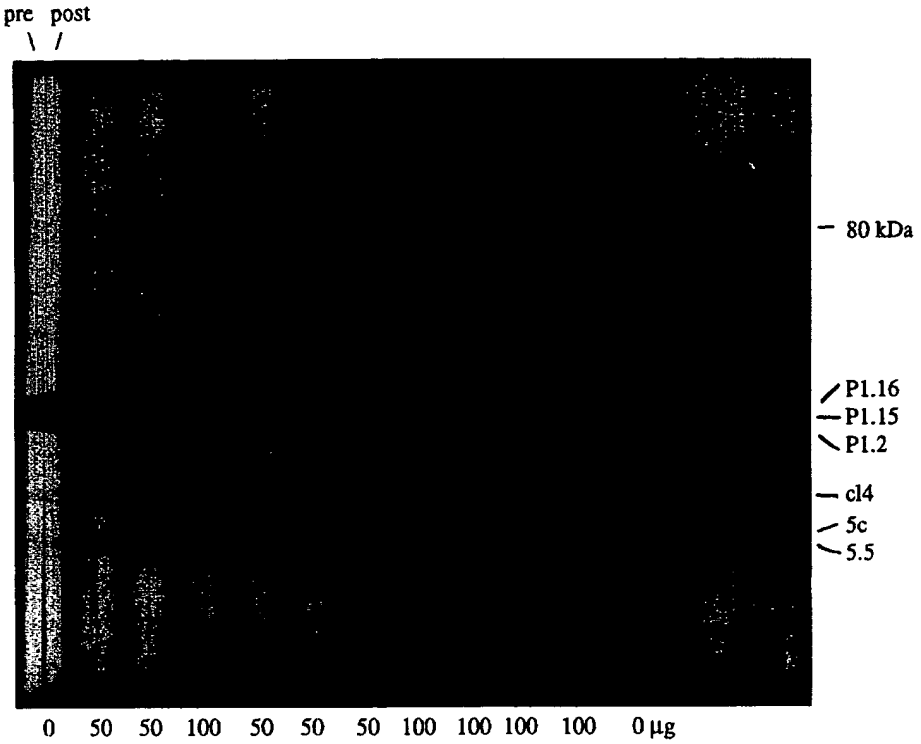

Figure 2 Antibody activity after one dosage of 50 or 100 μg hexavalent PorA meningococcal OMV vaccine in adult volunteers. Antibodies were measured by western blotting using a number of sera and trivalent PL16215 OMV. The location/presence of specific antigens was checked with monoclonals as indicated in the six most right lanes. The monoclonal antibodies are described in Materials and Methods. Pre- and postsera of vaccinees having received 0, 50, or 100 μg vaccine are indicated

observed (data not shown). *Table 1* shows the pre- and posttiters in the bactericidal test using the homologous test strain H44/76 (B:15:P1.7,16:L3) as well as the PorA and PorB negative mutants derived thereof[16]. Two volunteers revealed a fourfold increase in bactericidal titer against H44/76 which was absent when using the PorA negative mutant strain HI-5, pinpointing the PorA as target for these antibodies.

The increase in bactericidal titer was found to be serosubtype specific, when using strain 2996 (B:2b:P1.5,2;L3) as target strain no increases were found (data not shown). Four of the six volunteers did not reveal an increase in bactericidal titers after immunization.

Because of these results, our consecutive experiments were carried out with a hexavalent PorA containing OMV vaccine. The preparation of the vaccine is described in the companion paper[13]. Adult volunteers were immunized once and systemic and local side reactions were recorded (*Table 2*). Fever was not observed in any of the volunteers. Blood pressure and heart rate remained normal. Local pain at the injection site was more intense and of longer duration in the two groups administered vaccine in comparison to the placebo group. Of the volunteers who had been given the 50 μg vaccine two reported myalgia in the neck for 1 day a few days after vaccination, one volunteer reported a headache for half a day on the day following vaccination, and one reported gastro-intestinal complaints for 2 days 10 days after vaccination. Of the group administered the 100 μg vaccine one volunteer reported general malaise on the evening after vaccination; the symptoms had disappeared the following morning (*Table 2*).

Antibody induction was measured by ELISA using OMV, by western blotting, and by bactericidal assay. *Figure 1* shows antibody activity by using trivalent PorA containing OMV from strains PL16215, PL10124, and PL9146. Significant increases were recorded also by the nonvaccine OMV PL9146. *Figure 2* demonstrates antibody binding by western blotting, in addition to responses to PorA (class 1 OMP), responses were found against Rmp, Opa, Opc, and a 80 kDa OMP (for vaccine characterization, see Ref. 13). *Table 3* demonstrates the seroconversion in the bactericidal test, i.e. a fourfold increase or higher, against the six isogenic test strains. The 100 μg vaccine dose, i.e. 15 μg of each individual PorA, was found to reveal bactericidal seroconversion in approximately half of the volunteers against all six isogenic strains after one immunization. Most responses were found serosubtype-specific, i.e. PorA-specific, because not all isogenic strains were killed by the sera (*Table 4*). This was confirmed by using a PorA-negative mutant strain, which did not reveal any seroconversion patterns (data not shown). *Table 4* shows seroconversion of the volunteers using the bactericidal assay against the six isogenic strains used.

DISCUSSION

The human data with the purified OMP vaccine are in line with data from mice indicating the PorA to be an important target for bactericidal antibodies and secondly, these data suggested that purified OMP is not the optimal way to prepare meningococcal vaccines. In mice, OMV were found superior to purified OMP[2]. The

Table 3 Seroconversion (number with at least fourfold rise) in the bactericidal assay of adult volunteers after one dose of hexavalent PorA meningococcal OMV vaccine

Vaccine	Isogenic strains derived from H44/76					
	P1.7,16	P1.5,2	P1.19,15	P1.5[c],10	P1.12,13	P1.(7),4
Placebo	0	1	1	1	1	0
50 μg	4	3	3	4	6	2
100 μg	7	5	4	8	5	3

Number tested for each vaccine group: 10

Table 4 Seroconversion (≥fourfold increase in bactericidal titer) of volunteers immunized with 100 μg hexavalent PorA OMV vaccine

Volunteers	Test strain					
	P1.7,16	P1.5,2	P1.19,15	P1.5[c],10	P1.12,13	P1.7[h],4
1	+	+	−	+	−	−
2	+	−	−	+	−	+
3	+	−	+	+	+	+
4	+	+	−	+	+	−
5	−	+	−	+	+	−
6	−	−	−	−	−	−
7	−	−	−	−	−	−
8	+	+	+	+	−	−
9	+	+	+	+	+	+
10	+	−	+	+	+	−
	7/10	5/10	4/10	8/10	5/10	3/10

results with purified OMP vaccine in infants were also disappointing[8,9]. Unfortunately, OMV vaccines have not been tested in infants.

Since purified OMP vaccines appear to be suboptimal and the bactericidal antibodies induced mostly PorA serosubtype-specific, we developed a hexavalent PorA containing OMV vaccine[12,13]. The side-effects of the OMV vaccine appear to be related to local pain mostly. The vaccine was considered safe for further clinical evaluation. The vaccine contains *ca* 10% endotoxin on a weight basis of OMP. This endotoxin or LPS is part of the vesicle membrane and its pyrogenicity is within the range of acceptability of meningococcal polysaccharide vaccines[13]. The easily extractable LPS is removed by desoxycholate[17]. The LPS may add adjuvant activity to the vaccine as well as some relevant (i.e. bactericidal or endotoxin neutralizing) antibody inducing properties.

In order to evaluate the bactericidal antibody induction we constructed five isogenic strains in addition to strain H44/76 (B:15:P1.7,16), only differing in PorA. This allows antibodies with PorA and other specificities to be differentiated. PorA composes *ca* 90% of our vaccine, on a protein weight basis.

After one immunization with 50 or 100 μg total protein, seroconversion was measured with OMV-ELISA and western blotting. A number of antigens appeared to be involved such as PorA, Rmp, Opa, Opc, and a 80 kDa OMP. The nonvaccine OMV from strain PL9146[12] was found to bind similar amounts of antibodies as compared to vaccine OMV. By using the six isogenic test strains in the bactericidal assay, it was found that most bactericidal antibodies were PorA specific. This can be concluded from the specific patterns as well as from the negative results with PorA negative mutants. After one immunization approximately half of the volunteers showed a fourfold or higher increase in bactericidal antibody titers. Further evaluation of this vaccine will be undertaken in different age-groups using multiple immunization schedules. The purity of the vaccine can be further improved by deleting the Rmp Opa and Opc expression[12].

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